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Robert A. Silverman
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PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Group No.: 1646

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In re application of:

Sepp Kaul et al.

Serial No.: 09/864,954

Filed: May 24, 2001

For: **A NUCLEIC ACID WHICH IS UPREGULATED IN HUMAN TUMOR CELLS, A PROTEIN ENCODED THEREBY AND A PROCESS FOR TUMOR DIAGNOSIS**

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June 11, 2002

Assistant Commissioner for Patents
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Dear Sir:

Attached please find the certified copies of the foreign applications from which priority is claimed for this case:

Country	Application No.	Filing Date
Europe	00110953.7	May 26, 2000
Europe	00115369.1	July 15, 2000

Respectfully submitted,

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Bescheinigung Certificate Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr.	Patent application No.	Demande de brevet n°
	00115369.1	

Der Präsident des Europäischen Patentamts;
Im Auftrag

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Blatt 2 der Bescheinigung
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Page 2 de l'attestation

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A nucleic acid wich is upregulated in human tumor cells, a protein encoded thereby and a process for tumor diagnosis

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Case 20678 EP1

A nucleic acid which is upregulated in human tumor cells, a protein encoded thereby and a process for tumor diagnosis

Breast cancer is a major health problem since every eighth woman in Europe and the US succumbs to this disease (Moustafa, A.S., and Nicolson, G.L., Oncol. Res. 9 (1997) 505-525; Nicolson, G.L., Biochem. Soc. Symp. 63 (1998) 231-242). Treatment includes surgery, radiation, chemotherapy and combinations thereof, depending on the stage of the disease (Schwirzke, M., et al., Anticancer Res. 19 (1999) 1801-1814). A pronounced tropism of metastasis to the bones is characteristic for this disease starting with micrometastic lesions in the bone marrow which finally may outgrow to full-blown metastases. Bone metastasis result in bone fractures and spinal cord depression syndrome often followed by severe pain, aberrant calcium homeostasis and finally lead to death of the patients (Coleman, R.E., and Rubens, R.D., Br. J. Cancer 55 (1987) 61-66).

Analysis of genes involved in breast cancer and in cancer in general revealed a dichotomy with one category of genes with deregulated expression due to mutation and the other category of genes exhibiting changes in their regulation. These findings resulted in the grouping of cancer genes into two classes: class I genes are mutated or deleted, class II genes exhibit no alterations at the DNA level (Sager, R., Science 246 (1989) 1406-1412; Sager, R., Proc. Natl. Acad. Sci. USA 94 (1997) 952-955).

Summary of the Invention

In accordance with the present invention, a protein and the related gene, termed PKW, is provided which is upregulated in tumor cells, preferably in mammary tumor cells, as compared to their non-tumor counterparts. The PKW gene codes preferably for a polypeptide consisting of SEQ ID NO:2 or SEQ ID NO:4.

The present invention provides a nucleic acid which is upregulated in tumor cells, especially in mammary carcinoma cells, and which codes for a polypeptide which induces tumor progression or metastasis, the nucleic acid being selected from the group consisting of:

(a) SEQ ID NO: 1;

(b) a nucleic acid sequence which hybridizes under stringent conditions with a nucleic acid probe of the complementary sequence of (a);

(c) a nucleic acid sequence which, because of the degeneracy of the genetic code, is not a sequence of (a) or (b), but which codes for a polypeptide having exactly the same amino acid sequence as a polypeptide encoded by a sequence of (a) or (b); and

(d) a nucleic acid sequence which is a fragment of any of the sequences of (a), (b) or (c).

Preferably, the nucleic acid encodes a polypeptide consisting of amino acids of SEQ ID NO:2 or SEQ ID NO:4.

The present invention further provides a purified polypeptide having a sequence of amino acids SEQ ID NO:2 or SEQ ID NO:4.

The present invention further provides a process for detecting the presence or absence of at least one specific nucleic acid or mixture of nucleic acids, or distinguishing between two different sequences in said sample, wherein the sample is suspected of containing said sequence or sequences, which process comprises the following steps:

(a) incubating said sample under stringent hybridization conditions with a nucleic acid probe which is selected from the group consisting of:

(i) a nucleic acid sequence of SEQ ID NO:1 or a fragment thereof;

(ii) a nucleic acid sequence which is complementary to any nucleic acid sequence of (i);

(iii) a nucleic acid sequence which hybridizes under stringent conditions with the sequence of (i); and

(iv) a nucleic acid sequence which hybridizes under stringent conditions with the sequence of (ii); and

(b) determining whether said hybridization has occurred.

Moreover, the present invention provides a process for determining whether or not a test sample of tissue or fluid of a patient contains tumor cells or is derived from tumor cells, wherein the test sample and a second sample originating from non-tumor cells from the same individual or a different individual of the same species are used, which process comprises the following steps:

(a) incubating each respective sample under stringent hybridization conditions with a nucleic acid probe which is selected from the group consisting of:

(i) a nucleic acid sequence of SEQ ID NO: 1, or a fragment thereof;

(ii) a nucleic acid sequence which is complementary to any nucleic acid sequence of (i);

(iii) a nucleic acid sequence which hybridizes under stringent conditions with the sequence of (i); and

(iv) a nucleic acid sequence which hybridizes under stringent conditions with the sequence of (ii); and

(b) determining the approximate amount of hybridization of each respective sample with said probe, and

(c) comparing the approximate amount of hybridization of the test sample to an approximate amount of hybridization of said second sample to identify whether or not the test sample contains a greater amount of the specific nucleic acid or mixture of nucleic acids than does said second sample.

The invention further provides a method for the detection of mammary carcinoma as PKW is expressed only in mammary carcinoma cells or in metastatic cells thereof.

Detailed Description of the Invention

The present invention provides the new gene PKW, proteins coded thereby, and use of the PKW gene for diagnostics and therapeutics, especially in the field of cancer. In particular, the invention involves the identification of said gene PKW in tumor cells, especially in mammary carcinoma cells and tumor-cell-derived material such as DNA and RNA extracts from cells. The invention also relates to the detection of tumor cells and to gene therapy methods to modulate or inhibit PKW in its function in tumor cells.

The invention comprises a nucleic acid (PKW) which has upregulated expression in tumor cells and which is capable of inducing tumor progression and/or metastasis, especially in mammary carcinoma cells. The nucleic acid (PKW) has the sequence SEQ ID NO:1 or is a nucleic acid which, because of the degeneracy of the genetic code, differs from SEQ ID NO:1 and is preferably a nucleic acid which encodes the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4.

The invention further comprises recombinant polypeptides which are coded by the nucleic acid sequences according to the invention, preferably by the DNA sequence shown in SEQ ID NO:1 or fragments thereof preferably encoding the polypeptides of SEQ ID NO:2 or SEQ ID NO:4.

The invention further comprises recombinant expression vectors which are suitable for the expression of PKW, recombinant host cells transfected with such expression vectors, as well as a process for the recombinant production of a protein which is encoded by the PKW gene.

The invention further comprises a method for detecting a nucleic acid molecule of gene PKW, comprising incubating a sample (e.g., body fluids such as blood, cell lysates or a reverse transcript of an RNA sample) with the nucleic acid molecule according to the invention and determining hybridization under stringent conditions of said nucleic acid molecule to a target nucleic acid molecule for determination of presence of a nucleic acid molecule which is the PKW gene and therefore a method for the identification of tumor cells and preferably of mammary tumors. Quantitative detection can be performed by PCR techniques, preferably by the use of quantitative RT-PCR using, e.g., the LightCycler® of Roche Diagnostics GmbH, DE.

To determine whether a test sample contains tumor cells, the approximate amount of hybridization of the nucleic acid with the target nucleic acid or nucleic acids is determined. The approximate amount of hybridization need not be determined quantitatively, although a quantitative determination is encompassed by the present invention. Typically, the approximate amount of hybridization is determined qualitatively, for example, by a sight inspection upon detecting hybridization. For example, if a gel is used to resolve labelled nucleic acid which hybridizes to target nucleic acid in the sample, the resulting band can be inspected visually. When performing a hybridization of isolated nucleic acid which is free from tumor cells from an individual of the same species, the same protocol is followed. One can compare the approximate amount of hybridization in the test sample to the approximate amount of hybridization in the sample free from tumor cells, to identify whether or not the test sample contains a greater amount of the target nucleic acid or nucleic acids than does the sample which is free from tumor cells.

In a further method according to the invention no second sample is used. For the detection whether the expression of PKW gene is upregulated, the level of mRNA of PKW is compared with the level of mRNA of a standard gene (housekeeping gene (see, e.g., Shaper, N.L., et al., J. Mammary Gland Biol. Neoplasia 3 (1998) 315-324; Wu, Y.Y., et al., Acta Derm. Venerol. 80 (2000) 2-3) of the cell, preferably by RT-PCR.

For visual inspection in particular, it is recommended that an appreciable difference by visualized to assess that the test sample contains a greater amount of the target nucleic acid or nucleic acids.

As is shown in accordance with the present invention, the PKW nucleic acid is expressed in a greater amount in a tumor sample than in a sample free from tumor cells and/or in a greater amount than a housekeeping gene. A test sample containing tumor cells will have a greater amount of the PKW nucleic acid than does a sample which is free from tumor cells. To identify a test sample as containing upregulated PKW nucleic acid, i.e., wherein the cells are tumor cells or are tumor cells of a mammary carcinoma, it is preferable that the test sample have an approximate amount of PKW nucleic acid which is appreciably greater that the approximate amount in a sample free of tumor cells. For example, a test sample having an upregulated PKW gene may have approximately 15- to approximately 60-fold greater amount of PKW gene than a sample free of tumor cells or an at least 3-fold greater amount of PKW mRNA than mRNA of a housekeeping gene like glycerolaldehyde-3-phosphate dehydrogenase (GPDH) or porphobilinogen deaminase.

On the basis of the nucleic acids provided by the invention it is possible to provide a test which can be used to detect nucleic acids with upregulated expression in human tumor cells. Such a test can be carried out by means of nucleic acid diagnostics. In this case the sample to be examined is contacted with a probe that is selected from the group comprising

a) the nucleic acid sequence shown in SEQ ID NO:1, fragments thereof or a nucleic acid sequence which is complementary to one of these nucleic acid sequences, and
b) nucleic acids which hybridize under stringent conditions with one of the nucleic acids from a), wherein

the nucleic acid probe is incubated with the nucleic acid of the sample and the hybridization is detected optionally by means of a further binding partner for the nucleic acid of the sample and/or the nucleic acid probe. For obtaining a nucleic acid by hybridization in accordance with b), it is preferable to hybridize to a probe selected from the group of nucleic acids defined by the bases 724 to 1235 (small transcript) or bases 1831-2342 (large transcript) of SEQ ID NO:1, a fragment thereof of at least 200 bases or a sequence complementary thereto. Hybridization between the probe used and nucleic acids from the sample indicates the presence of the RNA of such proteins.

Methods of hybridization of a probe and a nucleic acid are known to a person skilled in the art and are described, for example, in WO 89/06698, EP-A 0 200 362, USP 2915082, EP-A 0 063 879, EP-A 0 173 251, EP-A 0 128 018.

In a preferred embodiment of the invention the coding nucleic acid of the sample is amplified before the test, for example by means of the known PCR technique. Usually a derivatized (labeled) nucleic acid probe is used within the framework of nucleic acid diagnostics. This probe is contacted with a denatured DNA, RNA or RT-DNA from the sample which is bound to a carrier and in this process the temperature, ionic strength, pH and other buffer conditions are selected - depending on the length and composition of the nucleic acid probe and the resulting melting temperature of the expected hybrid - such that the labeled DNA or RNA can bind to homologous DNA or RNA (hybridization see also Wahl, G.M., et al., Proc. Natl. Acad. Sci. USA 76 (1979) 3683-3687). Suitable carriers are membranes or carrier materials based on nitrocellulose (e.g., Schleicher and Schüll, BA 85, Amersham Hybond, C.), strengthened or bound nitrocellulose in powder form or nylon membranes derivatized with various functional groups (e.g., nitro groups) (e.g., Schleicher and Schüll, Nytran; NEN, Gene Screen; Amersham Hybond M.; Pall Biodyne).

Hybridizing DNA or RNA is then detected by incubating the carrier with an antibody or antibody fragment after thorough washing and saturation to prevent unspecific binding. The antibody or the antibody fragment is directed towards the substance incorporated during hybridization to the nucleic acid probe. The antibody is in turn labeled. However, it is also possible to use a directly labeled DNA. After incubation with the antibodies it is washed again in order to only detect specifically bound antibody conjugates. The determination is then carried out according to known methods by means of the label on the antibody or the antibody fragment.

The detection of the expression can be carried out for example as:

- in situ hybridization with fixed whole cells, with fixed tissue smears,
- colony hybridization (cells) and plaque hybridization (phages and viruses),
- Southern hybridization (DNA detection),
- Northern hybridization (RNA detection),
- serum analysis (e.g., cell type analysis of cells in the serum by slot-blot analysis),
- after amplification (e.g., PCR technique).

Therefore the invention also includes a method for the detection of carcinoma cells, comprising

a) incubating a sample of a patient suffering from cancer, selected from the group of body fluid, of cells, or of a cell extract or cell culture supernatants of said cells, whereby said sample contains nucleic acids with a nucleic acid probe which is selected from the group consisting of
   (i) the nucleic acid shown in SEQ ID NO:1 or a nucleic acid which is complementary to said sequence, and
   (ii) nucleic acids which hybridize with one of the nucleic acids from (i) and
b) detecting hybridization, preferably by means of a further binding partner of the nucleic acid of the sample and/or the nucleic acid probe or by X-ray radiography.

In addition, the invention comprises a process for determining whether or not a test sample originating from or containing human cells has a tumor progression potential, which process comprises the following steps:

(a) incubating a first compartiment of said sample under stringent hybridization conditions with a first nucleic acid probe which is selected from the group consisting of:
   (i) a nucleic acid with a sequence of SEQ ID NO:1 or a fragment thereof;
   (ii) a nucleic acid with a sequence which is complementary to any nucleic acid of (i);
   (iii) a nucleic acid with a sequence which hybridizes under stringent conditions with the nucleic acid of (i); and
   (iv) a nucleic acid with a sequence which hybridizes under stringent conditions with the nucleic acid of (ii); and
(b) incubating a second compartiment of said sample under stringent hybridization conditions with a second nucleic acid probe being a housekeeping gene or a fragment thereof;
(c) determining the approximate amount of hybridization of said sample with said first and second probe;
(d) identifying whether or not the test sample contains an at least 3-fold amount of nucleic acid hybridizing with the first probe in comparison to the amount of nucleic acid hybridizing with the second probe.

Preferably, the nucleic acid probe is incubated with the nucleic acid of the sample and the hybridization is detected optionally by means of a further binding partner for the nucleic acid of the sample and/or the nucleic acid probe. As probes, nucleic acids selected from the group consisting of nucleid acids defined by the bases 724 to 1235 (small transcript) or bases 1831-2342 (large transcript) of SEQ ID NO:1, a fragment thereof of at least 200 bases or a sequence complementary thereto are preferred.

The nucleic acids according to the invention are hence valuable markers in the diagnosis and characterization of tumors, especially of mammary tumors.

The invention further comprises a method for producing a protein whose expression is correlated with tumors, by expressing an exogenous DNA in prokaryotic or eukaryotic host cells and isolation of the desired protein, wherein the protein is coded by the nucleic acid molecules according to the invention, preferably by the DNA sequence shown in SEQ ID NO:1.

The protein can be isolated from the cells or the culture supernatant and purified by chromatographic means, preferably by ion exchange chromatography, affinity chromatography and/or reverse phase HPLC.

The invention further comprises a protein according to the invention which is encoded by a nucleic acid molecule according to the invention, preferably having the nucleotide sequence set forth in SEQ ID NO:1.

The present invention relates to the cloning and characterization of the gene PKW, which is especially characterized as a tumor progression gene, and as an upregulated gene indicative for the tumor progression potential of tumor cells, preferably of mammary tumor cells.

According to the invention inhibitors for the expression of PKW (e.g., antisense nucleotides) can be used to inhibit tumor progression, preferably of mammary carcinomas, in vivo.

The invention further provides methods for identifying and isolation of antagonists of PKW or inhibitors for the expression of PKW (e.g. antisense nucleotides). Such antagonists or inhibitors can be used to inhibit tumor progression and cause massive apoptosis of tumor cells in vivo.

According to the invention there are provided methods for identifying and isolation of PKW antagonists which have utility in the treatment of cancer. These methods include methods for modulating the expression of the polypeptides according to the invention, methods for identifying PKW antagonists which can selectively bind to the proteins according to the invention, and methods of identifying PKW antagonists which can modulate the activity of said polypeptides. The methods further include methods for modulating, preferably inhibiting, the transcription of PKW gene to mRNA. These methods can be conducted in vitro or in vivo and may make use of and establish cell lines and transgenic animal models of the invention.

A PKW antagonist is defined as a substance or compound which decreases or inhibits the biological activity of PKW, a polypeptide and/or inhibits the transcription or translation of PKW gene. In general, screening procedures for PKW antagonists involve contacting candidate substances with host cells in which invasiveness is mediated by expression of PKW under conditions favorable for measuring PKW activity.

PKW activity may be measured in several ways. Typically, the activation is apparent by a change in cell physiology, such as increased mobility and invasiveness in vitro, or by a change in the differentiation state, or by a change in cell metabolism leading to an increase of proliferation.

As shown in Fig 1, gene PKW is expressed only in one of the primary carcinoma cell lines, the one derived from the medullary mammary carcinoma. Topology of small and large transcripts of gene PKW as well as the potential proteins encoded by them are outlined schematically in Fig 2. Small and large transcript of gene PKW share 723 bp at the 5'end and 512 bp at the 3'end. The large transcript contains an insertion of 1107 bp (Fig 2A). The small transcript (bp 459-723 and 1831-1850 of SEQ ID NO:1) is due to differential splicing of the large transcript. The small transcript encodes a potential protein of 95 aa, the large transcript exhibits an open reading frame of 130 aa. Both potential proteins share an open reading frame of 88 aa with exception of one different aa at position 43 (nucleic acid position 586 of SEQ ID NO:1), followed by extensions of 7 aa and 42 aa for the smaller and the larger protein in different reading frames. The difference at position 43 may be a PCR artefact or could be caused by polymorphism. The 95 aa protein exhibits an isoelectric point (IEP) of 11.2, the IEP of the 130 aa protein is 10.4. These findings point to a nuclear localization of the proteins encoded by gene PKW. As shown in Fig 3 transcripts of gene PKW were detected only in salivary gland, not in other adult human tissues and in a small panel of embryonic tissues such as fetal brain, heart, kidney, liver, spleen, thymus and lung.

Promyelocytic leukemia cell line HL-60, HeLa cells, chronic myelogenous leukemia cell line K-562, lymphoblastic leukemia cell line MOLT-4, Burkitt lymphoma cell line Raji, colorectal adenocarcinoma cell line SW 480, lung carcinoma cell line A549 and melanoma cell line G361 all scored negative with respect to mRNA for gene PKW (Fig 3). The probe used for hybridization detects the small as well as the large transcript of gene PKW. A panel of mammary carcinoma cell lines described in (Schwirzke, M., et al., Anticancer Res. 18 (1998) 1409-1421) also tested negative with respect to the mRNA of gene PKW by Northern blotting as well as RT-PCR. These include MDA-435 (Cailleau, R., et al., In Vitro 14 (1978) 911-915) derived subclones 4C4 and 2A5, cell lines MDA-MB231 (Cailleau, R., et al., J. Natl. Cancer Inst. 53 (1974) 661-674), MDA-MB436 (Cailleau, R., et al., In Vitro 14 (1978) 911-915), ZR-75 (Engel, L.W., et al., Cancer Res. 38 (1978) 3352-3364), T47D (Freake, H.C., et al., Biochem. Biophys. Res. Commun. 101 (1981) 1131-1138), Hs578 T (Hackett, A.J., J. Natl. Cancer Inst. 58 (1977) 1795-1806), MCF-7 (Schiemann, S., et al., Anticancer Res. 17 (1997) 13-20; Schiemann, S., et al., Clin. Exp. Metastasis 16 (1998) 129-139), MCF-7_{ADR} (Schiemann, S., et al., Anticancer Res. 17 (1997) 13-20; Lee, J.H., et al., Biochem. Biophys. Res. Commun. 238 (1997) 462-467), LCC-1, LCC-2 and LCC-9 (Brünner, N., et al., Cancer Res. 53 (1993) 283-290; Brünner, N., et al., Cancer Res. 53 (1993) 3229-3232). In summary 11 mammary carcinomas were analyzed for expression of the small transcript of gene PKW by RT-PCR. 4 carcinomas scored positive. Parts of the results are displayed in Fig 4. Two of the positive carcinomas corresponded to ductal carcinomas and the other two matched with lobular carcinomas.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

SEQ ID NO:1 :	cDNA of PKW gene and amino acid sequence of large PKW splice variant.
SEQ ID NO:2 :	Amino acid of large PKW splice variant.
SEQ ID NO:3 :	cDNA and amino acid sequence of small PKW splice variant
SEQ ID NO:4 :	Amino acid of small PKW splice variant.
SEQ ID NO:5 :	Primer GSP1
SEQ ID NO:6 :	Primer GSP2
SEQ ID NO:7 :	Primer AUAP
SEQ ID NO:8 :	Primer RTR-5
SEQ ID NO:9 :	Primer RTF-6
SEQ ID NO:10 :	ß-actin reverse primer

SEQ ID NO:11 : ß-actin forward primer

Description of the Figures

Figure 1 Northern blot revealing differentially expressed mRNA's in cell lines derived from normal human mammary gland and from breast carcinomas of distinct stages of progression.
RNA was extracted from confluent cell lines, separated on a denaturing 1% agarose-formaldehyde gel, transferred to a positively charged nylon membrane and hybridized to an [α-^{32}P]-labeled probe corresponding to the appropriate subcloned fragment as revealed by the Differential Display Technique.
Lane a: HMEC, normal Human Mammary Epithelial Cells; lane b: cell line AR derived from medullary mammary carcinoma; lane c: cell line WA derived from invasive ductal mammary carcinoma; lanes d, e and f: cell lines 1590, HG15 and KM22, derived from mammary carcinoma bone marrow micrometastases; lane g: metastatic mammary carcinoma cell line KS, derived from malignant ascites fluid.

Figure 2 Schematic outline of transcripts of gene PKW as well as potential proteins encoded by these transcripts. Corresponding regions and domains are highlighted by conserved symbols.

Figure 3 Multiple Tissue Array
Normalized poly A+ RNA from different tissues and cell lines were hybridized to a 32P-labeled probe derived from gene PKW. E6 corresponds to 1 µg and H6 to 0.1 µg poly A^+ RNA from cell line AR. The code is revealed below.

Figure 4 Detection of transcripts of gene PKW in mammary carcinomas by RT-PCR.
RNA was extracted from mammary carcinomas and analyzed for transcripts corresponding specifically to the small transcript of gene PKW as described in Example 9.
Lane 0: DNA Molecular Weight Marker XIV (Roche Diagnostics GmbH, DE);
I: cell line AR; II-IX: different mammary carcinomas samples;

lane a: β actin control (2.5 µg RNA + specific primers for β actin); lane b: (2.5 µg RNA + specific primers for gene PKW); lane c: negative controls without RT: 2.5 µg RNA + specific primers for gene PKW.
An aliquot (15 µl) of the PCR products was analyzed on a 1.5% agarose gel. The bands specifically corresponding to mRNAs for gene PKW (137 bp) and ß-actin (587 bp) are depicted by arrows.

Example 1

Cell lines and cell culture

Human mammary epithelial cells (HMEC) were obtained from Bio Whittaker, Heidelberg, Germany. Primary mammary carcinoma cell lines AR and WA were obtained by fragmenting the primary tumor with scissors, treatment with collagenase (0.2 mg/ml in 5% FCS) and finally the cellular fraction was isolated by Ficoll gradient technique. Tumor cells were selected with a monoclonal antibody directed against MUC-1 coated to Dynabeads®(Dynal, Norway). MUC-1 antibodies are described in WO 99/40881. 5 x 10^7 beads were mixed with 10^7 cells, incubated for 1h at 4°C on a roller device, the bead fraction was collected on a magnet, washed twice with DMEM and finally the cells were propagated in 75cm^2 culture flasks in DMEM supplemented with 10%FCS. Two clones were identified after 4 weeks for both cell lines referred to as AR and WA. Cell line AR is derived from an invasive medullary mammary carcinoma, cell line WA is derived from an invasive ductal carcinoma. Cell lines 1590, HG15 and KM22 are derived from bone marrow micrometastases of mammary carcinoma patients. Cellular fraction was isolated on a Ficoll gradient, erythrocytes were lysed and the cells were suspended in DMEM + 10%FCS and tumor cells were isolated on Dynabeads® coupled with MUC-1 antibody as described above. The bead fraction was cultivated in DMEM + 10%FCS, 10 µg/ml insulin and 10 µg/ml transferrin. Outgrowth of tumor cell lines was observed after 8 weeks. Clones were isolated by treatment with EDTA and propagated under standard conditions as described above. Cell line KS was isolated from malignant ascites fluid of a mammary carcinoma patient. 2000 ml of ascites were collected and the cellular fraction was isolated on a Ficoll gradient. 2 x 10^7 cells were seeded into 750 cm^2 culture flasks. Tumor cell clusters were obtained from the culture supernatants in order to separate them from adherently growing fibroblasts and mesothelial cells (passages 1-4). Passages 5-10 resulted in cultures growing partly as a monolayer and in suspension. Finally cells were propagated as a monolayer in DMEM + 10% FCS.

Example 2

mRNA Differential Display PCR

Differential display reverse transcriptase polymerase chain reaction (DD-RT-PCR) was performed following the method described by Liang and Pardee using the RNAimage™ kits (GenHunter Corp. Brookline, MA) according to the manufacturer's protocol.

Total RNA was isolated from frozen cell pellets of all cell lines listed above making use of the RNeasy Midi® Kit (Qiagen, www.qiagen.de). Chromosomal DNA was removed from RNA samples by digestion at 37°C for 30 min with RNAse-free DNAse I using the MessageClean Kit® (GenHunter Corp. Brookline, MA).

RNA was used as a template for first strand cDNA synthesis in the presence of 3 different one-base anchored oligo-dT primers (H-T_{11}M, where M may be G, A or C).

For a 20 µl reaction, 1 µl of DEPC-treated H_2O, 4 µl of 5x reverse transcriptase buffer [125 mM Tris-Cl, pH 8.3, 188 mM KCl, 7.5 mM $MgCl_2$, 25 mM dithiothreitol (DDT)], 10 µl of dNTP mix [250 µM each], 2 µl of H-T_{11}M primer [2 µM], and 2 µl of DNA-free total RNA sample [0.1 µg/µl] were mixed. The solution was heated to 65°C for 5 min and cooled to 37°C for 10 min, and 1 µl [100 units] of Moloney murine leukemia virus (MMLV) reverse transcriptase was added. After incubation at 37°C for 1h, the reaction was terminated by incubation at 75°C for 5 min. The following PCR procedure was performed in a 20 µl reaction, containing 2 µl of reverse transcription reaction mixture, 9.2 µl of DEPC-treated H_2O, 2 µl pf 10x PCR buffer [100 mM Tris–Cl, pH 8.4, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatine], 1.6 µl of dNTP mix [25 µM each], 2 µl of the respective H-T_{11}M primer [2 µM], 2 µl of an arbitrary 13-mer primer, [2 µM], 1 µl of α-[^{35}S]dATP [>1000 Ci/mmole] and 0.2 µl of AmpliTag [10 units/µl] DNA polymerase (Perkin Elmer, Norwalk, CT). PCR included a total of 40 cycles at 94°C for 30s, 40°C for 2 min, 72°C for 30 s, and finally 5 min at 72°C. After adding 2 µl loading buffer to 3.5 µl of each sample, the PCR products of all cell lines were heated at 80°C for 2 min and loaded in parallel on a denaturing 6% polyacrylamide sequencing gel for electrophoresis. The dried gel was exposed to BioMax™ MR film (Kodak) at room temperature for 24 to 48 h and the autoradiogram was analyzed for the differentially expressed genes. Bands corresponding to cDNA's of interest reproducibly displayed in two independent DD-RT-PCR reactions were excised from the dried gel, and the cDNA was eluted from the gel by soaking the gel slice in 100 µl of dH_2O of 10 min and boiled for 15 min. After addition of 10 µl of 3M NaOAc and 5 µl glycogen [10mg/ml] as carrier the cDNA-fragments were recovered by precipitation

with 450 µl of ethanol and redissolved in 10 µl dH_2O. 4 µl eluted cDNA was reamplified in a second PCR using the same primer set and conditions except the dNTP concentrations of 20 µM each and no radioistope. As a control, gel slices were excised from lanes without visible bands on a level with the detected cDNA fragments of interest and treated as described above. The amplified PCR products obtained were analyzed on a 3% NuSieve® GTG (FMC BioProducts, Rockland), agarose gel, then purified using the QIAquick™ Gel Extraction kit (Qiagen, DE) and used as probes for Northern analysis.

Example 3

DNA sequencing of DD-RT-PCR fragments

All PCR fragments of interest were sequenced directly after extraction and purification from agarose gels. The nucleotide sequences data were analyzed for homologies with known genes or EST's in the current DNA data bases.

Example 4

Northern blot analysis

Poly A^+-RNA was isolated from total RNA. 1 µg of polyA^+-RNA from HMEC, AR, WA, 1590, KM22, HG15 and KS cells were loaded side by side on a denaturing 1% agarose formaldehyde gel and then size-separated by electrophoresis. Blotting to positively charged nylon membrane was done by capillary downward transfer. After UV-crosslinking (Stratagene UV Stratalinker™ 2400, www.stratagene.com) blots were hybridized. For that the DD-RT-PCR products were labeled with α-[^{32}P]dATP up to a specific activity of 2x 10^9 cpm/µg. Prehybridization (30 min) and hybridization (over-night) with radioactive probes were performed in ExpressHyb™ hybridization solution (Clontech, www.clontech.com) at 68°C according to the manufacturer's recommendation. Membranes were washed in solution 1 (2x SSC, 0.05% SDS) at room temperature for 30-40 min with continuous agitation and several replacements of the wash solution 1 followed by a washing step with solution 2 (0.1 x SSC, 0.1% SDS) at 50°C for 40 min with one change of fresh solution. The membranes were then exposed to Cronex™, Medical X-Ray Films (Sterling Diagnostic Imaging Inc., USA) at -80°C for 3 to 72 h. Equal loading and transfer of mRNA to the membrane was assessed by rehybridizing the blots with α-[^{32}P]dATP-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Example 5

Cloning of DD-RT-PCR fragments

Northern analysis was first performed using hybridization probes generated directly by PCR reamplification. Those amplified PCR fragments corresponding to differentially expressed mRNAs on a Northern blot were subcloned Subcloned fragments were isolated and stored for further experiments to verify differential expression.

Example 6

5′ RACE PCR

This method was applied to isolate the cDNA′s of gene PKW. To identify the 5′-sequences of both transcripts a 5′RACE (Rapid Amplification of cDNA Ends) PCR was performed following the manual as described in the 5′ RACE System for Rapid Amplification of cDNA Ends Kit, Version 2.0 (Gibco BRL, Life Technologies). First strand cDNA was synthesized from total RNA (without digestion with DNase I) using the gene-specific primer GSP1 (5′TTATCTTTATTCATTTTGG-3′, SEQ ID NO:5) and SuperScript™ II, an RNAse H⁻ derivative of the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MVL RT). After cDNA synthesis, the solution was purified from unincorporated dNTPs and GSP1. TdT (Terminal deoxynucleotidyl transferase) was used to add homopolymeric tails to the 3′ends of the cDNA. Tailed cDNA then was amplified by PCR using a nested, gene-specific primer GSP2(5′TGCGGGACTCGTCGTAAGTATGC-3′, SEQ ID NO:6),which anneals 3′ to GSP1, and the deoxyinosine-containing abriged universal amplification primer AUAP (5′GGCCACGCGTCGACTAGTAC-3′, SEQ ID NO:7). After several reactions with varying parameters also the longer cDNA (corresponding to the 2.6 kb transcript) could be detected in addition to the shorter one, which appears in any reaction. The enriched and purified cDNA′s were cloned and sequenced.

Example 7

Human Multiple Tissue Expression Array (MTE™)

This array (Clontech, Palo Alto, CA) contains normalized loadings of poly A^+-RNA from 76 different human tissues as well as control RNAs and DNAs as revealed in Fig 5. The blot was hybridized with an α-[^{32}P]dATP PKW cDNA according to the instructions of the manufacturer and exposed to X-ray film at –70°C.

Example 8

Isolation of RNA from Breast Tumor Tissues

Total RNA was isolated from frozen tumor samples. The frozen tissues were covered with the suggested amount of lysis buffer and immediately disrupted and homogenized by means of a homogenizer for 45-60 sec and 20000 U/min. The homogenized lysate was further processed as described in the manufacturer's protocol.

Example 9

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

In order to eliminate genomic DNA contamination the total RNA samples were treated with RNAse-free DNAseI at 37°C for 30 min. First strand synthesis was performed following the protocol of the first strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics GmbH, DE) by using the specific reverse primer RTR-5 (5'CCATTCATTCATTTTCAAG3', SEQ ID NO:8). The reverse transcription reaction was performed at 25°C for 10 min and then at 55°C for 60 min. After incubation, the AMV Reverse Transcriptase was denatured at 99°C for 5 min. For each sample a negative control reaction without AMV Reverse Transcriptase was performed.

The resulting single-stranded cDNA was amplified by PCR (High Fidelty PCR Master, Roche Diagnostics GmbH, DE) utilizing a second specific forward primer RTF-6 (5'AAAACGCATGGCTTGTC3', SEQ ID NO:9). The amplification was performed with an initial denaturation step at 94°C for 2 min, 10 cycles of 15 s denaturation at 94°C, 30 s annealing at 57°C and 1 min elongation at 72°C, followed by cycles under same conditions. Equal loading and integrity of mRNA was assessed by a control RT-PCR with β-actin primers (reverse primer 5'AGGGTACATGGTGGTGCCGCCAGAC3' SEQ ID NO:10 forward primer 5'CCAAGGCCAACCGCGAGAAGATGAC3' SEQ ID NO:11).

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Patent Claims

1. A nucleic acid which is upregulated in mammary tumor cells, said nucleic acid being selected from the group consisting of:

   (a) SEQ ID NO: 1;
   (b) a nucleic acid sequence which hybridizes under stringent conditions with a nucleic acid probe of the complementary sequence of (a);
   (c) a nucleic acid sequence which, because of the degeneracy of the genetic code, is not a sequence of (a) or (b), but which codes for a polypeptide having exactly the same amino acid sequence as a polypeptide encoded by a sequence of (a) or (b); and
   (d) a nucleic acid sequence which is a fragment of any of the sequences of (a), (b) or (c).

2. A nucleic acid according to claim 1, having a sequence which codes for a polypeptide of amino acids of SEQ ID NO:2 or SEQ ID NO:4.

3. The nucleic acid according to claim 1, wherein the nucleic acid sequence of (b) hybridizes in 6.0 X SSC at 45°C to 55°C, followed by a wash with from about 2.0 X SSC to about 0.2 X SSC at a temperature from about 50°C to about 65°C.

4. The nucleic acid according to claim 1, wherein the nucleic acid has a sequence of nucleotides 459 to 848 of SEQ ID NO:1 or of nucleotides 1 to 285 of SEQ ID NO:3.

5. An expression vector comprising a nucleic acid of claims 1 to 4.

6. A host transformed by a nucleic acid of claims 1 to 4.

7. A polypeptide which induces tumor progression, wherein said polypeptide is encoded by a nucleic acid selected from the group consisting of:

   (a) SEQ ID NO: 1;
   (b) a nucleic acid sequence which hybridizes under stringent conditions with a nucleic acid probe of the complementary sequence of (a) ; and
   (c) a nucleic acid which is a fragment of any of the sequences of (a) or (b).

8. The polypeptide according to claim 7, wherein the nucleic acid sequence of (b) hybridizes in 6.0 X SSC at 45°C to 55°C, followed by a wash with from about 2.0 X SSC to about 0.2 X SSC at a temperature from about 50°C to about 65°C.

9. A process for detecting the presence or absence of at least one specific nucleic acid or mixture of nucleic acids, or distinguishing between two different nucleic acids in said sample, wherein the sample is suspected of containing said nucleic acid or acids, which process comprises the following steps in order:

(a) incubating said sample under stringent hybridization conditions with a nucleic acid probe which is selected from the group consisting of:
  (i) a nucleic acid with a sequence taken from the group consisting of SEQ ID NO:1 or a fragment thereof;
  (ii) a nucleic acid with a sequence which is complementary to any nucleic acid of (i);
  (iii) a nucleic acid with a sequence which hybridizes under stringent conditions with the nucleic acidof (i); and
  (iv) a nucleic acid with a sequence which hybridizes under stringent conditions with the nucleic acid of (ii); and
(b) determining whether said hybridization has occurred.

10. The process in accordance with claim 9, wherein said hybridization conditions comprise 6.0 X SSC at 45°C to 55°C, followed by a wash with from about 2.0 X SSC to about 0.2 X SSC at a temperature from about 50°C to about 65°C.

11. A process for determining whether or not a test sample originating from or containing human cells has a tumor progression potential, wherein a second sample originating from non-tumor cells from the same individual or a different individual of the same species is also used, which process comprises the following steps:

(a) incubating said samples under stringent hybridization conditions with a nucleic acid probe which is selected from the group consisting of:
  (i) a nucleic acid with a sequence of SEQ ID NO:1 or a fragment thereof;
  (ii) a nucleic acid with a sequence which is complementary to any nucleic acid of (i);
  (iii) a nucleic acid with a sequence which hybridizes under stringent conditions with the nucleic acid of (i); and

(iv) a nucleic acid with a sequence which hybridizes under stringent conditions with the nucleic acid of (ii); and

(b) determining the approximate amount of hybridization of each respective sample with said probe and

(c) comparing the approximate amount of hybridization of the test sample to an approximate amount of hybridization of said second sample to identify whether or not the test sample contains a lower amount of the nucleic acid than does said second sample.

12. A process for determining whether or not a test sample originating from or containing human cells has a tumor progression potential, which process comprises the following steps:

(a) incubating a first compartiment of said sample under stringent hybridization conditions with a first nucleic acid probe which is selected from the group consisting of:

(i) a nucleic acid with a sequence of SEQ ID NO:1 or a fragment thereof;

(ii) a nucleic acid with a sequence which is complementary to any nucleic acid of (i);

(iii) a nucleic acid with a sequence which hybridizes under stringent conditions with the nucleic acid of (i); and

(iv) a nucleic acid with a sequence which hybridizes under stringent conditions with the nucleic acid of (ii); and

(b) incubating a second compartiment of said sample under stringent hybridization conditions with a second nucleic acid probe being a housekeeping gene or a fragment thereof;

(c) determining the approximate amount of hybridization of said sample with said first and second probe;

(d) identifying whether or not the test sample contains an at least 3-fold amount of nucleic acid hybridizing with the first probe in comparison to the amount of nucleic acid hybridizing with the second probe.

Abstract

A nucleic acid molecule (PKW) with the nucleic acid sequence SEQ ID NO:1 is upregulated in mammary carcinoma cells. The PKW protein of SEQ ID NO:2 and SEQ ID NO:4 is also provided. A process for determining whether a sample contains tumor cells is provided.

Fig.1

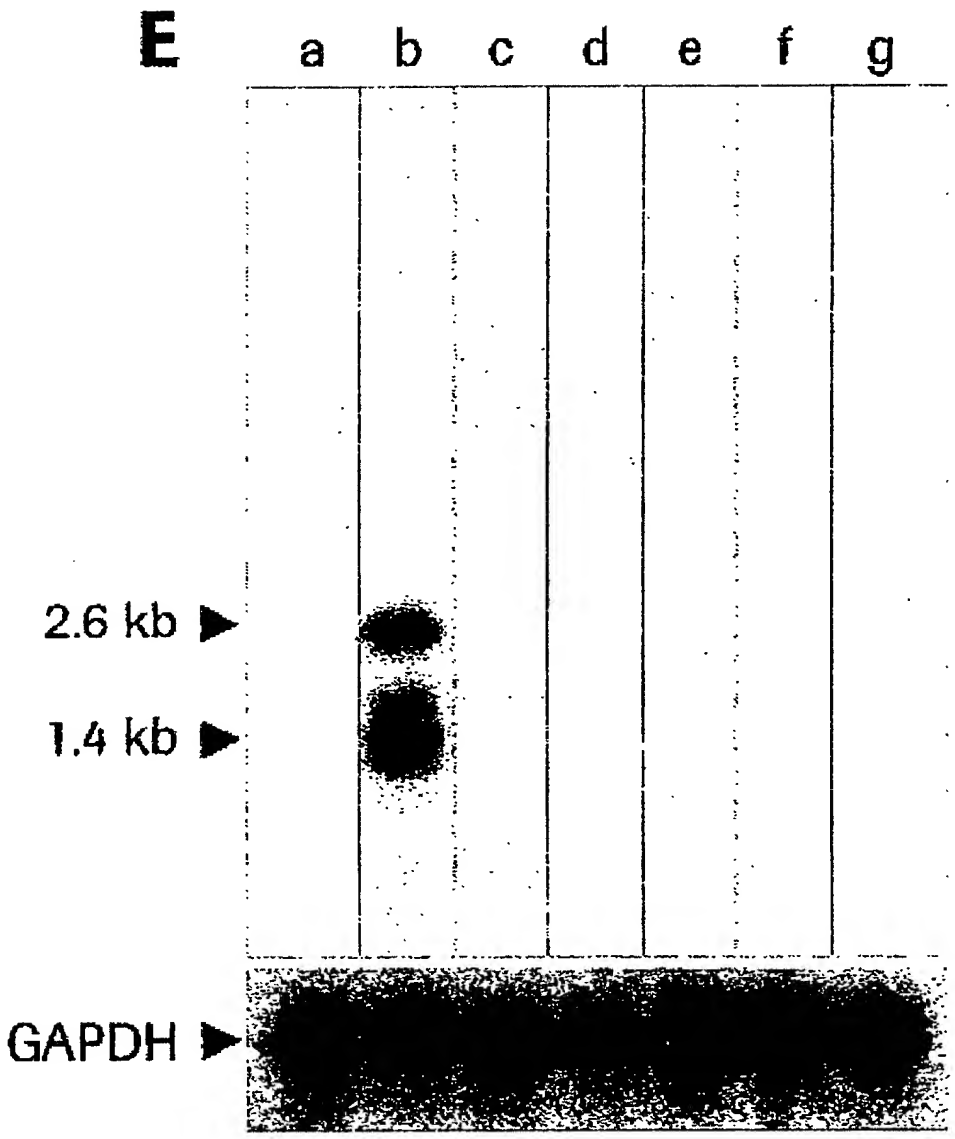

Fig.2

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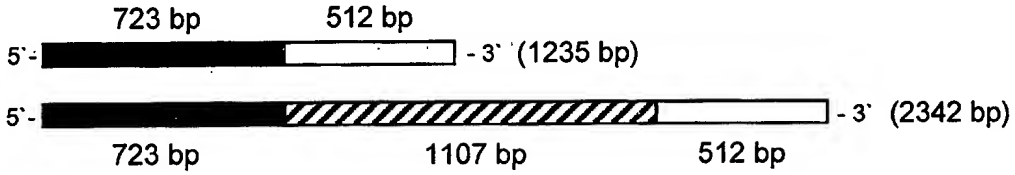

Large transcript of gene PKW

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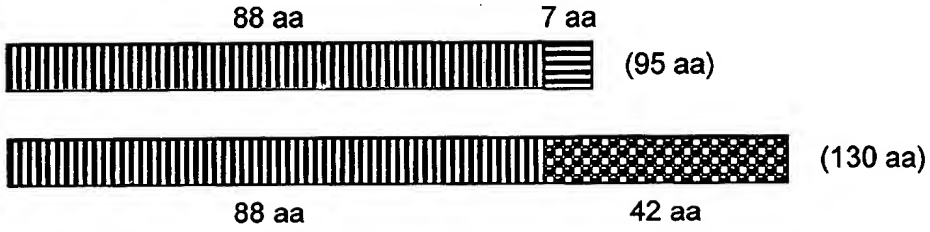
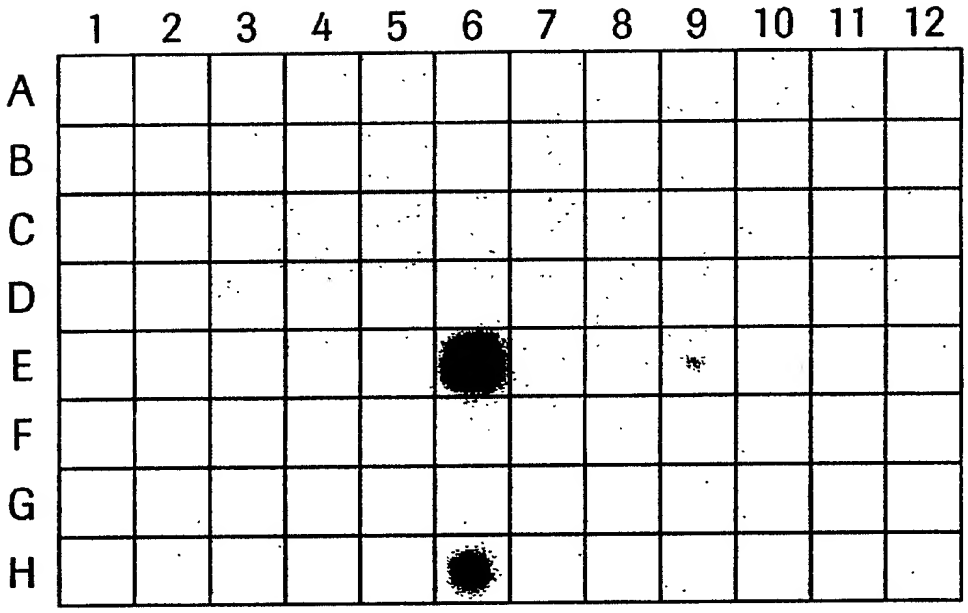
Protein encoded by small transcript of gene PKW



Protein encoded by large transcript of gene PKW

Fig.3



	1	2	3	4	5	6	7	8	9	10	11	12
A	whole brain	cerebellum, left	substantia nigra	heart	esophagus	colon, transverse	kidney	lung	liver	leukemia, HL-60	fetal brain	yeast total RNA
B	cerebral cortex	cerebellum, right	accumbens nucleus	aorta	stomach	colon, desending	skeletal muscle	placenta	pancreas	HeLa S3	fetal heart	yeast tRNA
C	frontal lobe	corpus callosum	thalamus	atrium, left	duodenum	rectum	spleen	bladder	adrenal gland	leukemia, K-562	fetal kidney	*E. coli* rRNA
D	parietal lobe	amygdala	pituitary gland	atrium, right	jejunum		thymus	uterus	thyroid gland	leukemia, MOLT-4	fetal liver	*E. coli* DNA
E	occipital lobe	caudate nucleus	spinal cord	ventricle, left	ileum		peripheral blood leukocyte	prostate	salivary gland	Burkitt's lymphoma, Raji	fetal spleen	Poly r(A)
F	temporal lobe	hippo-campus		ventricle, right	ilococum		lymph node	testis	mammary gland	Burkitt's lymphoma, Daudi	fetal thymus	human C_0t-1 DNA
G	p.g.* of cerebral cortex	medulla oblongata		inter-ventricular septum	appendix		bone marrow	ovary		colorectal adeno-carcinoma SW480	fetal lung	human DNA 100 ng
H	pons	putamen		apex of the heart	colon, ascending		trachea			lung carcinoma, A549		human DNA 500 ng

* paracentral gyrus

Fig.4